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PRODUCTION OF DENGUE 2 ENVELOPE PROTEIN IN THE YEAST SACCHAROMYCES CEREVISIAE

FINAL, PHASE I REPORT

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The four serotypes of dengue viruses are a leading cause of morbidity throughout the tropics and subtropics. For use in diagnostics and in subunit vaccines, a reliable and inexpensive source of dengue antigens is required. We have evaluated the ability of the yeast *Saccharomyces cerevisiae* to express antigenic dengue envelope (E) glycoprotein. We subcloned the E gene of dengue-2 strain PR159S1 into six *S. cerevisiae* expression vectors which encompass both constitutive and regulated promoters, with and without secretion signal peptide-encoding sequences. We have screened by Western blots both intracellular andsecreted proteins of yeast transformed with the recombinant yeast-dengue genes for antigenic dengue E protein. Our results demonstrate that the yeast *S. cerevisiae* will express and secrete antigenic dengue E glycoprotein.

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INTRODUCTION AND OBJECTIVES

Dengue virus is a positive sense, single-stranded RNA virus transmitted to man by mosquitoes. The four dengue serotypes as a group are probably the most important viruses transmitted to man by arthropods, causing millions of cases of dengue fever annually. Typical dengue infection results in an acute illness marked by fever, headache, and aching joints. In children, however, dengue infection can be more severe, resulting in potentially fatal dengue hemorrhagic fever-dengue shock syndrome (DHF/DSS).

The live attenuated yellow fever vaccine strain 17D is the prototypical flavivirus vaccine. Attempts to develop live attenuated dengue vaccine strains have been for the most part unsuccessful, although live attenuated strains of DEN-1, DEN-2, and DEN-4 are being tested still in Thailand, and the US Army is continuing to test live attenuated vaccine-candidate DEN-2 and DEN-4 strains. Most live attenuated candidate vaccine strains have either been under- or over-attenuated.

A complicating factor in dengue vaccine development is that immunity against dengue of one serotype does not confer protection against dengue of the other serotypes. Additionally, Halstead (1982) has demonstrated that anti-dengue antibodies can enhance virus infectivity *in vitro*, and it has been proposed that infection-enhancing antibodies can function *in vivo* resulting in DHF/DSS (Halstead, 1981).

Owing to the lack of success in developing live attenuated vaccine strains, many labs have focused on developing subunit dengue vaccines or recombinant live viral vaccines. Subunit vaccines may eventually include synthetic dengue peptides or recombinant dengue proteins expressed in microorganisms, and live viral vectors such as vaccinia may express *in vivo* immunogenic dengue peptides.

During the herein reported Phase I SBIR research, we proposed to test the feasibility of expressing the DEN-2 PR159S1 envelope glycoprotein in the yeast *Saccharomyces cerevisiae*. For these studies we used the cDNA clone C8 of Hahn *et al* (1988). Phase I technical objectives were the following: 1) Subclone a cDNA encoding the DEN2 PR159S1 E protein gene into six yeast expression vectors. The six expression vectors combine DNA elements for constitutive and regulated expression and for intracellular and secreted expression. 2) Transform appropriate yeast strains with the expression constructs, and 3), using Western blots, screen for expression of antigenic DEN2 E protein, determine relative levels of DEN2 E protein expressed by the various expression plasmids, and examine the distribution of the E protein between whole cell extracts and culture medium.

Our results demonstrate *S. cerevisiae* will express and secret antigenic DEN2 envelope protein. The results also suggest that the DEN2 E protein is glycosylated when translated with a secretion signal peptide. Expression was achieved with both a constitutive promoter and an inducible promoter, although protein expressed from the inducible constructs apparently is glycosylated more slowly than E protein expressed from the constitutive promoter. In a Western blot format, the yeast-expressed DEN2 E protein is recognized by human anti-flavivirus antibodies.

MATERIALS AND METHODS

Expression vectors. *S. cerevisiae* expression vectors used in these studies were developed at and provided by SmithKline and Beecham. All the vectors contain the origin of replication from the endogenous yeast 2μ plasmid and the *TRP1* gene. The 2μ origin of replication maintains a high copy number (30-50) of the plasmid per cell, the *TRP1* gene provides a selectable marker. In addition, all six plasmids contain sequences derived from the *Escherichia coli* plasmid pBR322, which provide an origin of replication in *E. coli* and the gene encoding resistance to ampicillin. All six of the vectors have at least three cloning sites downstream of the promoter or secretion signal encoding sequences, followed by translational termination signals in all three reading frames.

To control transcription, the plasmids have one of two *S. cerevisiae* promoters: either the strong constitutive promoter from the glyceraldehyde phosphate dehydrogenase (GAPDH) gene, or the copper-inducible copper metallothionein (*CUP1*) promoter. All six plasmids use the 3' sequences from the *S. cerevisiae* cytochrome C (*CYC1*) gene for transcriptional termination and polyadenylation signals. One pair of vectors was designed for intracellular expression, a second pair contains the *Candida albicans* glucoamylase (glucoamy) secretion signal, and the third pair contains the alpha mating factor (MFα) leader peptide. The glucoamylase secretion signal peptide is 20 amino acids long, and its *Xma*I cloning site (used in these studies) is within the nine nucleotides encoding the following three amino acids. Alpha factor is a small (13 amino acid) peptide hormone synthesized as a 165 amino acid precursor containing an 89 residue leader and four alpha factor units, each separated by a spacer peptide. The alpha factor leader peptide is complex. An initial 20 amino acid secretion signal peptide is followed by 69 amino acids which are glycosylated and which are removed by the joint action of the *STE13* and *KEX2* gene products. The *KEX2*-encoded protease cuts on the carboxyl side of the peptide sequence lys-arg (amino acids 84 and 85), after which the *STE13*-encoded dipeptidyl amino-peptidase removes glu-ala dipeptide repeats from the amino terminus of the remaining protein. Expression vectors pLS5 and pLS6 encode just one glu-ala dipeptide (residues 86 and 87) following the kex2 cleavage site. The *Bgl*II cloning site used in these studies is

within the nine nucleotides downstream of those encoding the glu-ala dipeptide. The relevant properties of the vectors are summarized in the table below.

S. cerevisiae Expression Vectors

Plasmid	Promoter	Secretion Signal	Cloning Sites
pYSK137	GAPDH	None	*XhoI NcoI SalI*
pYSK138	*CUP1*	None	*XhoI NcoI SalI*
pYSK140	*CUP1*	Glucoamy	*XmaI SmaI NcoI SalI*
pYSK142	GAPDH	Glucoamy	*XmaI SmaI NcoI SalI*
pLS5	GAPDH	MFα	*StuI BglII NcoI SalI*
pLS6	*CUP1*	MFα	*StuI BglII NcoI SalI*

Yeast Strains, Culturing, and Transformation. The genotypes of the two *S. cerevisiae* yeast strains (G. Livi, personal communication) used in these experiments are:

F762 - *MATa ura3-52 trp1^1*

GL43 - *MATa ura3-52 trp1^1 proA::URA3*

F762 was used for secretion of dengue E protein. GL43 contains a genetically engineered "disruption" mutation of *PROA*. The encoded enzyme, ProA, a major vacuolar protease, is believed to be responsible for processing carboxypeptidase Y and other vacuolar hydrolases into their mature, active form (Woolford, et al, 1986). Thus, intracellular, heterologous proteins are more stable in GL43. Both strains contain a deletion mutation of *TRP1* to prevent reversion to prototrophy.

Standard protocols for culturing yeast and extracting DNA Sherman et al (1983) were followed. Transformation of yeast used the lithium acetate treatment to prepare transformation-competent cells (Ito et al, 1983). Yeast transformants were selected as tryptophan prototrophs on synthetic minimal medium plus uracil (SD+Ura). Cells harboring the inducible expression plasmids were induced by adding $CuSO_4$ to a final concentration of 0.2 mM.

Dengue cDNA. cDNA clone C8 of DEN2 PR159S1 (Hahn et al, 1988) was used as envelope (E) gene source material, and was provided by Dr. E. Henchal (Walter Reed Army Institute of Research). This plasmid contains a randomly primed cDNA, cloned using *Eco*RI linker oligonucleotides into the *Eco*RI site of pGEM1 (Promega Biotech), and contains most of the capsid (C) gene, all of the premembrane (PreM), membrane (M), and E structural protein genes and extends into the first nonstructural protein (NS1) gene.

Polymerase Chain Reaction. We generated the DEN2 E gene fragment subcloned in these studies using the polymerase chain reaction (PCR) and DEN2-specific oligonucleotide primers designed with restriction enzyme recognition sites for subsequent cloning. The Taq polymerase and reagents used for PCR were provided in the GeneAmp kit (Perkin Elmer/Cetus) and were used according to the directions of the manufacturer. Oligonucleotides were synthesized under subcontract by the University of Hawaii Biotechnology Support Facility.

Protein Gels and Western Blots. Proteins were prepared for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) from small cultures (5-20 ml) grown at 30° with agitation and harvested during exponential growth (10^6 to 10^7 cells per ml). To analyze protein secreted into the culture medium, cells were harvested by centrifugation and the resulting media supernatants were filtered through 0.45 μM Acrodisc low protein binding filters (Gelman). Culture media were then concentrated by centrifugation in Centricon 30 concentrators (Amicon), and were prepared for SDS-PAGE by adding one fifth volume of 5X sample buffer (1X sample buffer = 3% SDS, 10% glycerol, 5% β-mercaptoethanol, 125 mM Tris, pH 6.8) and heating at 100° for 5 min.

Cellular proteins for SDS-PAGE were prepared in one of three ways. Total cellular proteins were prepared by resuspending washed cell pellets in 1X sample buffer, incubating 5 min at 100°C, and spinning down cell debris. Soluble cellular proteins were prepared by resuspending washed cell pellets in TEN (10 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl) + 0.6 mM PMSF, vortexing vigorously (five 20 sec pulses, stored on ice between pulses) in the presence of acid washed glass beads (425-600 μm), and separating the supernatant from cell debris by centrifugation. One fifth volume of 5X sample buffer was added to the cleared supernatant and heated at 100° for 5 min. Membrane associated and insoluble cell proteins were obtained from the remaining cell debris by adding 1X sample buffer and heating at 100° for 5 min. Amounts of sample representing protein from culture medium or cellular proteins derived from 0.5 ml of culture were loaded on all protein gels.

Standard methods for protein gels (Laemmli, 1970) and immunoblots (Towbin, 1979) were followed. The amount of culture medium or cellular proteins loaded represented that obtained from 0.5 ml of culture. Proteins were separated on 10% SDS-PAGE gels, and either directly visualized by silver staining (SIGMA), or transferred electrophoretically to nitrocellulose membranes and detected immunologically. Hyperimmune anti-DEN2 mouse ascites fluid, anti-E monoclonal antibody, and pooled anti-flavivirus human sera were a gift from Dr. E. Henchal. Dengue antibody negative human serum was a gift from Dr. Leon Rosen (Dept. of Trop. Med., Univ. Hawaii). To detect the primary mouse and human

antibodies, goat anti-mouse or goat anti-human antibodies (Promega), respectively, conjugated to alkaline phosphatase were used as secondary antibodies in combination with alkaline phosphatase substrates. Culture medium and cellular proteins from yeast transformed with nonrecombinant vectors were used as negative antigen controls. Total protein from DEN3-infected C6/36 mosquito cells was used as a positive antigen control.

Immunoprecipitation. For immunoprecipitation, cells were grown in synthetic medium lacking methionine and labeled with Trans^{35}S-label™ (ICN). Cells containing constitutive expression plasmids were grown to midexponential phase, and cultured for 2 1/2 hours further in the presence of label. Cells containing inducible vectors were induced at mid log, grown for 1 hour, and labeled for 2 1/2 hours. Cells were harvested by centrifugation, and media were concentrated with Centricon 30 concentrators. An equal volume of 2X RIPA (1X RIPA = 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Na deoxycholate, 1% Triton-X100, and 0.1% SDS) was added to media samples. Washed cells were resuspended in 1X RIPA + 0.6 mM PMSF, and broken by vortexing in the presence of glass beads.

Pooled anti-flavivirus human sera were diluted 1:10 with unlabeled nonrecombinant cellular or media proteins prepared in the same manner as labeled proteins, and incubated overnight at 4°. 500,000 (cellular proteins) or 10,000 (media proteins) cpm of TCA precipitable counts were then added to the preabsorbed sera and incubated at 4° for 24-48 h. Antigen-antibody complexes were collected by adding a 10% suspension of fixed *Staphylococcus aureus* cells (SIGMA), incubating 10 min at room temperature, and centrifuging for 15 min. Pelleted materials were washed twice in phosphate buffered saline (PBS), resuspended in 1X Laemmli buffer, and heated at 100° prior to SDS-PAGE analysis.

RESULTS

DEN-2 E Gene Subcloning. To subclone the DEN-2 strain PR159S1 E gene cDNA fragment, we PCR-amplified the desired sequences using dengue sequence specific oligonucleotide primers with appended adaptor sequences (see below). Plasmid C8, Hahn et al (1988) containing cDNA of most of the C gene, all of PreM, M and E, and part of NS1 was linearized and used as a template for PCR. The primers for PCR synthesis of the E gene fragment are presented below. Adaptor sequences are in lower case, and dengue sequences are in upper case. The primer names indicate from which dengue gene the sequence is derived, the nucleotide position of the first dengue nucleotide in the oligonucleotide, and whether the primer directs plus (p) or minus (m) strand synthesis. Also shown are the positions of restriction endonuclease cleavage and the direction of translation (->).

```
                 XhoI XmaI  SmaI BglII
                   v    v    v    v
D2E937p    gcctcgagcc cgggagatct ATG CGC TGC ATA GGA ATA TC
                                 Met Arg Cys Ile Gly Ile ->

                  SalI
                   v
D2E2271m   gcgtcgacta CCC GTA GAT TGC TCC AAA AAC C
                     End Gly Tyr Ile Ala Gly Phe Val <-
```

The multiple restriction endonuclease sites in the plus strand primer enabled cloning the same PCR-generated fragment into all six yeast expression vectors. The *Sal*I site at the 3' end of the fragment was used to clone into all expression vectors. The ATG codon in D2E937p corresponds to the first codon of the DEN2 E gene and its inclusion serves to initiate translation for the intracellular-expression vectors. For the secretion-expression vectors, the *Xma*I and *Bgl*II sites were positioned to fuse the E gene in frame with the secretion signal peptide encoding sequences. Primer D2E2271m was designed to exclude the membrane anchor at the carboxy end of the E protein, which could otherwise prevent secretion of the E protein. Based on the model of Mandl et al (1989), the membrane anchor comprises approximately the 50 carboxy terminal amino acids, which our construction eliminates.

PCR amplifications using clone C8 (Hahn et al, 1988) as template generated an expected 1.3 kilobase (kb) fragment (data not shown). To aid screening *E. coli* transformants, the PCR-amplified DNA was cloned into vector pVZ1 (Henikoff and Eghtedarzadeh, 1987), which provides the blue-white screen based on β-galactosidase inactivation, prior to subcloning into the six yeast expression vectors. Two pVZ1-DEN2 E gene clones (pVZD2ER and pVZD2ES) were obtained and both were subcloned into the yeast expression vectors. DNA sequence analysis of both ends of these two DEN2 E gene clones confirmed the identity of both clones as DEN2 E sequences. This sequencing, performed simultaneously with the expression studies presented below, detected two single nucleotide deletions at the carboxy end of pVZD2ER, whose effects are to replace four dengue amino acids with 17 random amino acids. The 3' ends of the two fragments and the encoded proteins' carboxy ends are given below. The DNA sequence shown stops at the *Sal*I site used for subcloning into the yeast expression vectors. The *Sal*I site used for subcloning from pVZD2ER, the additional nucleotides, and the stop codon originate in the multiple cloning site sequence of pVZ1. The nucleotide position indicated is in reference to the sequence of DEN2 PR159S1. The carboxy ends of clones derived from pVZD2ES are:

2251 GTT TTT GGA GCA ATC TAC GGG TAG TCGAC
Val Phe Gly Ala Ile Tyr Gly End

Whereas, the carboxy ends of clones derived from pVZD2ER are:

2251 GTT TTT GGA GAA TCT ACG GGT AGT CGC GCC GGA ATT CGA GCT
Val Phe Gly Glu Ser Thr Gly Ser Arg Ala Gly Ile Arg Ala

CGG TAC CCG GGG ATC CTC TAG AGTCGAC
Arg Tyr Pro Gly Ile Leu End

Subcloning into Yeast Expression Vectors. Appropriate restriction endonuclease fragments from pVZD2ES and pVZD2ER were subcloned into the six yeast expression vectors. Available restriction endonuclease sites in the vectors are presented in the Materials and Methods. *Xho*I-*Sal*I fragments were subcloned into vectors pYSK137 and pYSK138, *Xma*I-*Sal*I fragments were subcloned into vectors pYSK140 and pYSK142, and *Bgl*II-*Sal*I fragments were subcloned into pLS5 and pLS6. These recombinant yeast-dengue gene containing plasmids were named to reflect the expression vector and the pVZD2E clone used; e.g. pY137D2ES contains the DEN2 E gene fragment from plasmid pVZD2ES inserted into vector pYSK137.

Yeast Transformations. The genotype of yeast strains used is shown in Materials and Methods. Strain GL43 contains a genetically engineered mutation in the *PROA* gene to provide greater stability of intracellularly expressed heterologous proteins, and it was used for transformations involving derivatives of vectors pYSK137 and pYSK138. Strain F762, which is isogenic with strain GL43, was used for all other plasmids.

Transformants were selected for their ability to grow on medium lacking tryptophan. Transformants will be referred to by the plasmid which they contain, with a decimal number (e.g. pY137D2ES.1) indicating individual isolates. All transformants were unstable for the Trp$^+$ phenotype as expected for extra-chromosomal inheritance of the *TRP1* gene, thus ruling out the possibility of gene conversion of the chromosomal *trp1* deletion mutation. Duplicate Southern blots of total DNA (data not shown) prepared from yeast transformants grown in selective medium (i.e. lacking tryptophan) were probed with a radiolabeled DEN2 E gene fragment and a *TRP1* gene fragment. With one exception, all DEN2 E gene fragments, whose size varied with particular constructs, were of the expected sizes. (The single transformant with an aberrant E gene fragment expressed no E protein [datum not shown].) The *TRP1* fragments were of constant and proper size.

Expression of DEN2 E Protein in Yeast. Proteins from yeast cultures transformed with nonrecombinant and dengue-recombinant plasmids were screened by Western blots for proteins recognized by polyclonal mouse hyperimmune ascites fluid (MHAF). Cellular

proteins were prepared either as total cellular or as soluble and membrane associated fractions, and proteins in the culture supernatant were concentrated by centrifugation in Centricon 30 concentrators (see Materials and Methods). Proteins from transformants with the constitutive GAPDH promoter-DEN2 E gene fusions were prepared from cells in midexponential phase growth. Transformants containing the inducible *CUP1* promoter-DEN2 E gene fusions were induced during midexponential growth, and proteins were prepared at various times following induction.

Protein from yeast containing plasmid pY137D2ER (constitutive, intracellular) contained two bands of molecular weights ~50 kilodaltons (kd) reactive with MHAF (Fig. 1, lane 2). The same preparation can be seen in Fig 2 (lane 14) beside total cellular protein from a pYSK137 nonrecombinant transformant (lane 15). The E protein in the yeast expression vectors was engineered to lack the 50 carboxy terminal amino acids corresponding to the membrane anchor. The proteins expressed by pYSK137 and pYSK138 also lack a secretion signal peptide, and consequently are expected not to enter the secretion pathway and should remain unglycosylated. Both of the antigenic bands ran on 10% SDS-PAGE faster than E protein and slower than NS1 protein from DEN3 strain CH53489-infected C6/36 mosquito cells. Based on amino acid sequence alone, DEN2 E produced by pY137D2ER is expected to be approximately 50.4 kd (= 458 amino acids x ~110 daltons/amino acid; subclones derived from pVZD2ES should encode a protein of size ~49.0 kd = 445 amino acids x ~110 daltons/amino acid). Proteins from two pY138D2ES (inducible, intracellular) yeast transformants were examined up to 14 h after induction for DEN2 E protein, but no antigenic protein was found.

Secretion of recombinant proteins from plasmids pYSK140 (inducible) and pYSK142 (constitutive) is based upon the *C. albicans* glucoamylase secretion signal peptide. Figure 1, lanes 3-5 contain proteins secreted into the culture medium from three pY142D2E transformants. This secreted form is of very high apparent molecular weight (>80 kd). Secreted protein from one of these transformants is faintly visible on Figure 2, lane 3; no antigenic material was seen in the culture medium of the pYSK142 nonrecombinant (Fig 2, lane 5). The intracellular form expressed from pY142D2E is of a lower molecular weight than the secreted form and corresponds to that expected for this construction (~55 kd, Fig 2, lane 11). Intracellular antigenic proteins expressed by pY140D2E (Fig 2, lane 12) after four hours of induction comprised a doublet and were of lower molecular weight than that observed in the constitutively expressing pY142D2E recombinants. These may represent forms of E which have not been as extensively glycosylated as the form seen in the constitutively expressing pY140D2E recombinants. The absence of E protein in the culture medium of pY142D2E transformants may reflect insufficient time for export of the induced protein.

Secretion of proteins from cells transformed with pLS5D2E (constitutive) and pLS6D2E (inducible) recombinants uses the alpha factor leader peptide. A variation in size similar to that seen for pY140D2E and pY142D2E can again be seen for pLS5D2E and pLS6D2E. pLS6D2E transformants contain two antigenic forms of the same approximate size as found in pY142D2E transformants, whereas intracellular antigenic material found in pLS5D2E is seen in a single predominant band (~66 kd, Fig 2, lane 8) of greater molecular weight than in pLS6D2E transformants. The corresponding bands are not observed in protein from the pLS5 and pLS6 nonrecombinant transformants (Fig 2, lanes 10 and 7). It appears that the two lower molecular weights are associated with the induced expression of E, and that the single higher molecular weight is found in constitutively expressing cells. This suggests that a precursor product relationship exists among the three forms, and that the time of induction (4 h) is inadequate to generate the 66 kd form. Because these proteins were engineered to enter the secretion pathway, the probable difference among the three forms is the extent of glycosylation.

The distribution of E protein among the culture medium, soluble cellular proteins, and the membrane associated proteins (see Materials and Methods) was analyzed for the secreted forms of E. For pLS5D2E, the majority of constitutively expressed antigen is intracellular and was not soluble in buffer lacking detergent (Fig 3, lanes 4-9; Fig 4, lanes 4-6). The same association of E protein with the cellular membrane fraction is also apparent for pY140D2E (Fig 3, lane 10-15). Following 4 h induction of cells bearing the pY142D2E recombinant, E proteins can be seen as a triplet of approximate sizes 55, 60, and 68 kd. These sizes are consistent with the idea that the lower molecular weight forms represent intermediates in the process of glycosylation. These experiments do not address proteolytic processing of the alpha factor leader peptide, which is complex (see above), nor do they address the possibility of proteolytic degradation. Although proteolytic processing or degradation of the E protein can not be ruled out, and in fact removal of the secretion signal peptide is desired, such events would not result in increasing molecular weight.

The effect of the time of induction on the molecular weight of E protein expressed by pLS6D2E (alpha factor leader peptide) and pY140D2E (glucoamylase secretion signal) can be seen in Fig 4 (lanes 2 and 3). In these two lanes, two molecular weight forms can be seen after 21 h of induction. The two bands, ~55 and 66 kd, are larger than the forms seen in cultures induced for only 4 h (Fig. 3, lane 14; Fig 2, lanes 6 and 12), in which the largest form is approximately equal in size to the smallest form seen after 21 h induction. The increase in molecular weight with time of induction is consistent with the idea proposed above of gradually increasing glycosylation. It is noteworthy that the precursor bands are absent from the proteins of constitutive expressers. Their occurrence in the proteins of induced expres-

sers may be due to the toxic effect of cupric ions on cell physiology. It is also apparent that both bands expressed by pLS6D2ER are slightly larger than the two expressed by pY140D2ES. These may be due to the known sequence difference at the carboxy end of the peptide, which is 13 amino acids longer in the pVZD2ER-derived subclones than in the pVZD2ES-derived subclones (see above).

The reactivity of yeast-expressed DEN2 E protein with anti-flavivirus human antibodies in a Western blot format is shown in Fig. 4 (lane 2). Fig. 4, lane 1 presents the detection of E and NS1 proteins from dengue-infected C6/36 cells using pooled human anti-flavivirus sera. The reaction with NS1 is stronger than with E. Lane 2 was divided between two panels. The left panel was probed with the human sera, and the right panel with hyperimmune mouse anti-DEN2 ascites fluid. It can be seen that the same two protein bands react with both human and mouse sera.

The results of immunoprecipitations of DEN2 E protein expressed in yeast were not available at the time of writing this Phase I final report. We hope to include these data with the Phase II proposal to be submitted soon.

ESTIMATES OF TECHNICAL FEASIBILITY

These results support the technical feasibility of expressing the envelope protein of dengue viruses in the yeast *S. cerevisiae*. We demonstrated the production of antigenic material using both a constitutive promoter and an inducible promoter. The observation of larger molecular weight forms for those constructs involving a secretion signal than for those which involve no secretion signal supports the interpretation that the DEN2 E protein is glycosylated in yeast. Following induction, gradually increasing molecular weight forms for the cell-associated DEN2 E linked to either the glucoamylase or the alpha factor secretion signals supports the idea that the protein is being glycosylated.

The experiments performed to assess the extra- versus intracellular location of DEN2 E protein found the majority of the protein cell associated. In the experiments performed during Phase I, we examined cells during exponential growth to avoid the changes in physiology that may occur when cells enter stationary phase. Brake et al (1984) found, however, that human epidermal growth factor secreted from yeast using the alpha factor leader peptide did not reach maximum levels (>95%) until 24 h following entry into stationary phase. If true for DEN2 E, this would certainly enhance the amount of E protein that is secreted. Further experimentation would be required to assess this for the DEN2 E protein-yeast recombinants. Additionally, we compared the amount of protein in equivalent proportions of the total (i.e., protein from 0.5 ml of culture medium and from the cells in that

same volume). Examination of the abundance of E protein in the culture medium on a per weight of total secreted protein may provide a more favorable assessment of its expression and secretion.

It is apparent from the figures that multiple forms of the E protein are found in the intracellular proteins of yeast. We have proposed that these forms represent different extents of glycosylation of the E protein, and that the lower molecular weight forms represent precursors to the higher molecular weight forms. There are experimental approaches to addressing this. Pulse chase experiments could establish the precursor product relationship among the various forms, and could document the time required for synthesis and secretion. Treatment of the proteins with endoglycosidases followed by sizing on protein gels would establish whether the observed size differences can be attributable to differences in the peptide itself.

We have documented that one of the two PCR-generated E gene clones had two single nucleotide deletions at the carboxy terminal end of the DNA fragment. It is obviously desirable to avoid such clones in production of authentic, antigenic proteins. Although the protein produced by the two fragments appeared to behave similarly except for a possible slight size difference, we deem it a requirement to verify the complete sequence of any clone used for production of antigens or immunogens. Secretion of the E protein may be improved if we consider an even smaller fragment. We have removed the 50 carboxy terminal amino acids in the present constructs. Analysis of the hydrophobicity (Fig. 5) of the E protein lacking the anchor reveals that its carboxy end comprising about 30 amino acids is still hydrophobic, which may serve to keep the protein membrane associated. This amino acid sequence corresponds to the stick of the "lollipop"-like model of the tertiary structure of the E protein of tick-borne encephalitis (Mandl et al, 1989) and does not overlap the potential T-cell determinant. If not detrimental to the antigenicity and immunogenicity of the E protein, removal of an additional 30 amino acids from the carboxy end may enhance secretion of the DEN2 envelope glycoprotein from yeast.

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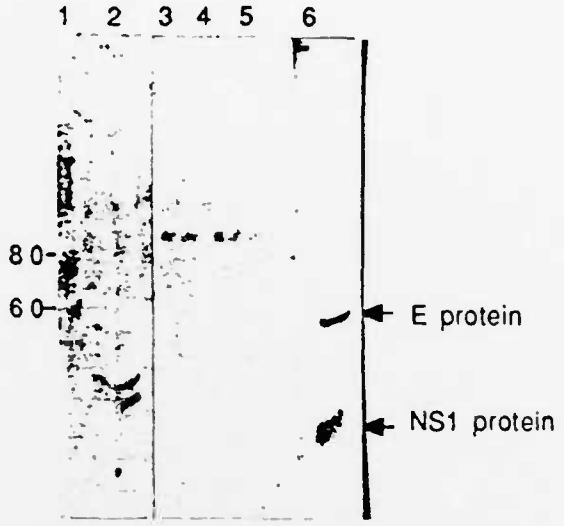

Figure 1. Detection of intracellular and secreted DEN2 Envelope protein. Transformed yeast were grown for four hours past mid-log phase and proteins were analyzed by Western Blotting. After pelleting the cells, the medium was filtered and concentrated in Centricon-30 concentrators, adjusted to 1X sample buffer, and boiled for five minutes. Proteins were separated on a 10% acrylamide gel, electroblotted to nitrocellulose and probed with hyperimmune mouse ascites fluid. The lanes contain: Lane 1) prestained molecular weight markers, sizes are indicated in kilodaltons. Lane 2) total cellular proteins from the non-secretor pY137D2ER.1 (constitutive promoter). Lanes 3, 4, and 5) concentrated medium from .5 ml of culture of pY142D2ES.2 (constitutive promoter, glucoamylase secretion signal), pY142D2ES.1, and pY142D2ER.2. Lane 6 contains total protein from Dengue 3-infected C6/36 mosquito cells; the E protein and NS1 protein are indicated.

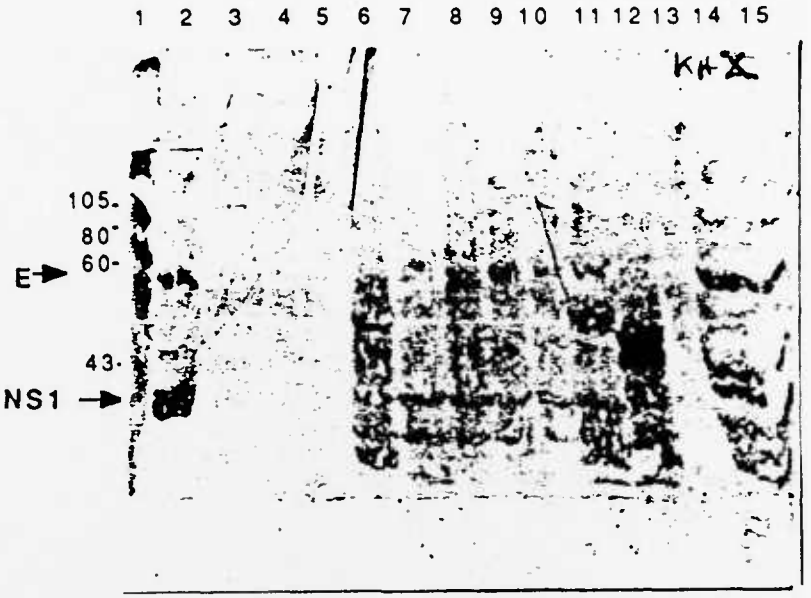

Figure 2. Survey of yeast-expressed DEN2 E protein. Proteins from yeast and culture medium were prepared as described in Materials and Methods and analyzed by Western blot using anti-DEN2 mouse hyperimmune ascites fluid as probe. The lanes contain: Lane 1) prestained molecular weight markers, sizes are shown in kilodaltons. Lane 2) total cellular protein from Dengue 3-infected C6/36 mosquito cells. The E protein and NS1 protein are indicated. Lanes 3, 4, and 5) culture media proteins from pY142D2ES.2 (constitutive promoter, glucoamylase secretion signal), pY142D2ER.2 and the nonrecombinant pYSK142. Lanes 6 and 7) total cellular protein from pLS6D2ES.2 (inducible promoter, alpha factor secretion signal) and the nonrecombinant pLS6at 4 hours post induction. Lanes 8, 9, and 10) total cellular protein from pLS5D2ES.1 (constitutive promoter, alpha factor secretion signal) and pLS5D2ER.1 and the nonrecombinant pLS5. Lane 11) total cellular protein from pY142D2ER.2 (constitutive promoter, glucoamylase secretion signal). Lane 12) total cellular protein from pY140D2ES.2 (inducible promoter, glucoamylase secretion signal) at four hours post induction. Lane 13) blank. Lane 14) total cellular protein from pY137D2ER.1 (constitutive promoter, intracellular producer). Lane 15) total cellular protein from the nonrecombinant pYSK137.

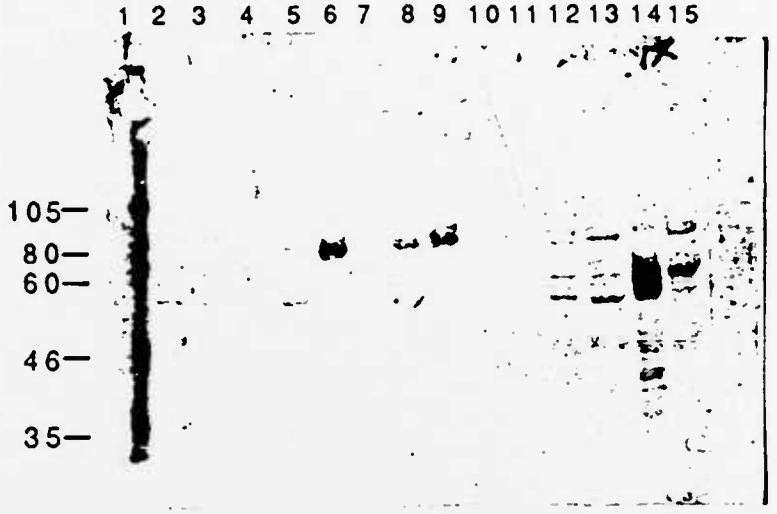

Figure 3. Compartmentalization of E protein. Cells were harvested 4 hours after reaching mid-log phase and proteins were analyzed by Western Blotting. Cells containing inducible constructs were induced at mid-log (t = 0 hours). Proteins were separated on 10% polyacrylamide, electroblotted to membrane and probed with hyperimmune mouse ascites fluid. The lanes contain: Lane 1) prestained molecular weight markers, sizes are indicated in kilodaltons. Lane 2) soluble cellular proteins of pLS5 non-recombinants (constitutive promoter, alpha factor secretion signal). Lane 3) membrane associated proteins from the nonrecombinant pLS5. Lanes 4, 5, and 6) proteins from the culture medium, soluble cellular, and membrane fractions respectively of pLS5D2ES.2 (constitutive promoter, alpha factor secretion signal). Lanes 7, 8, and 9) proteins from the medium, soluble cellular, and membrane fractions of pLS5D2ER.2 . Lanes 10 and 11) culture medium proteins of pY140D2ER.1 (inducible promoter, glucoamylase secretion signal) at 4 and 0 hours post induction. Lanes 12 and 13) soluble cellular proteins from pY140D2ER.1 at 4 and 0 hours post induction. Lanes 14 and 15) membrane associated proteins from pY140D2ER.1 at 4 and 0 hours post induction. Some sample leaked from lane 14 into lane 15 at the time of loading.

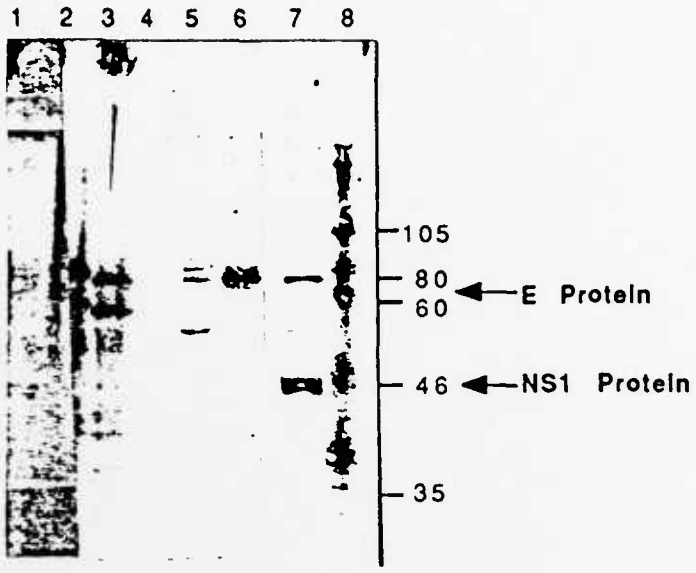

Figure 4. Compartmentalization and Effect of time of induction on expression of DEN2 E. A panel of the blot containing lane 1 and a portion of lane 2 were probed with pooled human antiflavivirus antisera. The remaining blot containing lanes 2 through 8 was probed with hyperimmune mouse ascites fluid. Lane 1 contains total cellular protein from Dengue 3-infected C6/36 mosquito cells. Lane 2 contains total cellular protein from pLS6D2ER.1 (inducible promoter, alpha-factor secretion signal) harvested 21 hours post induction. Lane 3 contains total cellular proteins from pY140D2ES.2 (inducible promoter, glucoamylase secretion signal) harvested 21 hours post induction. Lanes 4, 5, and 6 contain medium proteins, soluble cellular material, and membrane associated material from pLS5D2ES.2 (constitutive promoter, alpha factor secretion signal), harvested at mid-log phase. Lane 7 contains total cellular protein from Dengue 3-infected C6/36 mosquito cells, the E protein and NS1 proteins are indicated. Lane 8 contains prestained molecular weight markers, sizes are indicated in kilodaltons.

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Hydrophilicity Plot

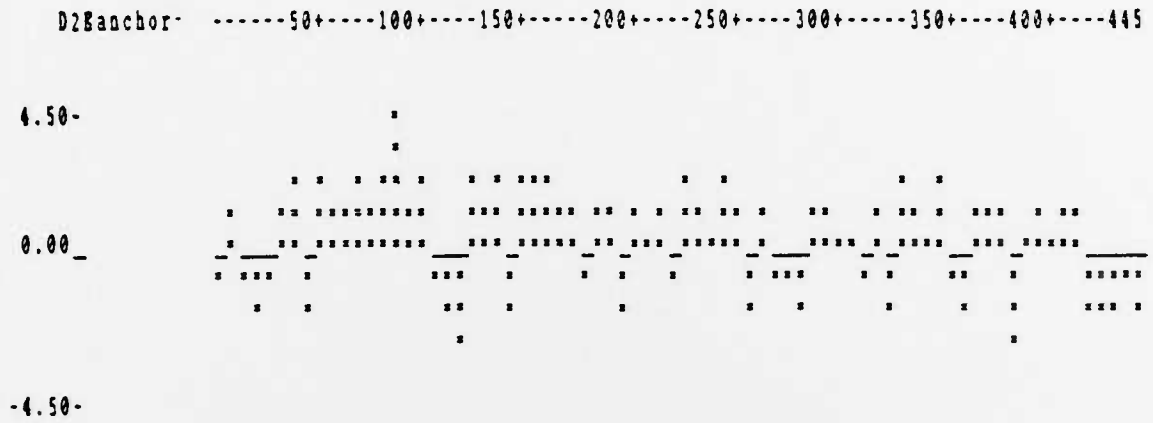

Figure 5. Hydrophilicity analysis of DEN2 E protein. The hydrophilicity of the E protein minus the 50 carboxy terminal amino acids comprising the membrane anchor was analyzed by the IBI Pustell sequence analysis programs. Positive scores and negative scores represent domains which are on average hydrophilic or hydrophobic, respectively.